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SSR markers associated with fertility restoration genes against *Triticum timopheevii* cytoplasm in *Triticum aestivum*

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Summary

Heterosis is an important way to improve yield and quality for many crops. Hybrid rice and hybrid maize contributed to enhanced productivity which is essential to supply enough food for the increasing world population. The success of hybrid rice in China has led to a continuous interest in hybrid wheat, even when most research on hybrid wheat has been discontinued in other countries for various reasons including low heterosis and high seed production costs. The *Timopheevii* cytoplasmic male sterile system is ideal for producing hybrid wheat seeds when fertility restoration lines with strong fertility restoration ability are available. To develop PCR-based molecular markers for use in marker-assisted selection of fertility restorer lines, two F_2 populations derived from crosses R18/ND36 and R9034/ND36 were used to map fertility restoration genes in the two elite fertility restorer lines (R-lines) R18 and R9034. Over 678 SSR markers were analyzed, and markers closely linked to fertility restoration genes were identified. Using SSR markers, a major fertility restoration gene, *Rf3*, was located on the 1B chromosome in both populations. This gene was partially dominant in conferring fertility restoration in the two restorer lines. SSR markers Xbarc207, Xgwm131, and Xbarc61 are close to this gene. These markers may be useful in marker-assisted selection of new restorer lines with *T. timopheevii* cytoplasm. Two minor QTL conferring fertility restoration were also identified on chromosomes 5A (in R18) and 7D (in R9034) in two R-lines.

Introduction

Heterosis is an important way to improve yield and quality for many crops. Hybrid cultivars have been developed to take advantage of heterosis in the production of many field crops such as rice, maize, sorghum, sunflower, oil seed rape, and cotton. In the production of hybrid seeds, several systems can be used to eliminate self-pollination of female lines including: application of chemical hybridization agents, mechanical removal of anthers or male flowers, or use of cytoplasmic or nuclear-encoded male sterility. Cytoplasmic male sterility (CMS), a maternally inherited condition in which a plant carrying mitochondrial defects is unable to produce functional pollen, has been observed in numerous species (Bentolila et al., 2002). Nuclear genes called restorers of fertility (*Rf*) have the ability to suppress the male-sterile phenotype and restore production of pollen to plants carrying the deleterious mitochondrial cytoplasm. CMS lines are widely used for hybrid seed production in important widely grown crops such as rice, sorghum and sunflower (Perez-Prat & van Lookeren Campagne, 2002).

Rice is a staple food in China. Hybrid rice produced with CMS has a yield advantage of about 15–20% over the best commercial pure-line rice varieties. Since 1984, the area planted to hybrid rice in China increased to about 50% of the total rice area of the country

(Yuan, 1992; Lu & Hong, 1999). Through the cultivation of hybrid rice, China has continuously increased its internal rice production, and this has allowed the country to reduce the area assigned to rice, thus allowing more land to be used for other production activities. The successful exploitation of hybrid rice in China has encouraged hybrid production of wheat – another major food crop in the world (Zhang et al., 2001).

CMS and fertility restoration in wheat were first discovered by Kihara (1951) and Fukasawa (1955). In 1962, Wilson and Ross (1962) reported complete CMS in common wheat induced by substituting its cytoplasm with *T. timopheevii* cytoplasm. Since then CMS has been used widely for the production of hybrid wheat. In this system, three lines are required: a restorer line (R-line) carries Rf genes with *T. timopheevii* cytoplasm, a maintainer line (B-line) carries common wheat nuclear and cytoplasm, and a male sterile line (A-line) carries the same nuclear genes as the B-line but with *T. timopheevii* cytoplasm. Seven *Rf* genes have been reported to restore the fertility against *T. timopheevii* cytoplasm, and their chromosome locations have been determined, namely, *Rf1* (1A), *Rf2* (7D), *Rf3* (1B), *Rf4* (6B), *Rf5* (6D), *Rf6* (5D), and *Rf7* (7B) (Tahir & Tsunewaki, 1969; Yen et al., 1969; Bahl & Maan, 1973; Du et al., 1991). Ma et al. (1991) transferred an *Rf* gene from *Aegilops umbellulata* to wheat. This gene was located on chromosomes 6AS and 6BS.

The R-line is crucial in hybrid wheat breeding using CMS with *T. timopheevii* cytoplasm because it not only recovers hybrid fertility but also determines hybrid vigor (Wilson, 1968). Elite R-lines should have strong and stable fertility restoration ability and high general combining ability for important agronomic traits (Done, 1973; Jost & Glatki-Jost, 1978; Zhou et al., 2000). Breeding R-lines is a process of improving agronomic traits and transferring *Rf* genes. Using traditional breeding technology, breeders need to test fertility restoration ability of selected lines annually beginning with the F_3. In the following generation, the fertility of the testcross hybrids represents the restoration ability of the tested lines, and this fertility restoration ability should be investigated before selection for other traits (Johnson & Patterson, 1973; Ghiast & Lucken, 1982; Zhou et al., 1999). The necessity of doing fertility testcross procedures adds more work to R-line selection and impedes the breeding efficiency of R-line development.

Use of molecular markers provides a more accurate approach than traditional methods for manipulating genes of agronomic importance such as fertility restoration. Restriction fragment length polymorphisms (RFLPs) have been used to map *Rf* genes against *T. timopheevii* cytoplasm in wheat (Ma & Sorrells, 1995; Kojima et al., 1999). Simple sequence repeat (SSR, also termed microsatellite) markers are PCR-based and more chromosome specific in wheat than AFLPs (Bryan et al., 1997; Röder et al., 1998); therefore, SSRs are suitable molecular markers for marker-assisted selection of R-lines. Our objective in this study was to identify SSR markers linked to *Rf* genes against *T. timopheevii* cytoplasm in two elite R-lines, R18 and R9034.

Materials and methods

Plant materials

Development of R18 and R9034

Figure 1 shows the schemes of selection process of R9034 and R18. The detailed information on their breeding history is described later.

Jiangsu Academy of Agricultural Science, P.R. China, has had a continued hybrid wheat breeding program since Professor Cai Xu introduced a set of A-line, B-line, and R-line with *T. timopheevii* cytoplasm in 1967. The male-sterile and fertile restorer lines were crossed and backcrossed continuously with local varieties from Jiangsu province to produce a series of male-sterile, maintainer, and restorer lines adapted to the ecological conditions of Jiangsu province. Yuanxuan 124 (a derivative from Kansas restorer) was crossed with Primepi, and progeny from that cross were crossed with Oleson Dwarf with the objective of decreasing plant height. After continuous selection for several years, a

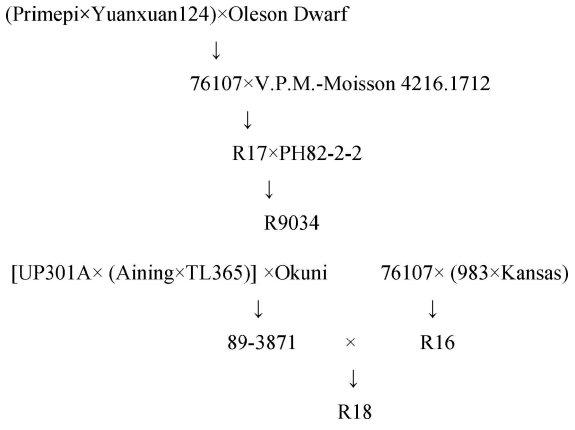

Figure 1. Schemes for breeding process of R9034 and R18.

fertility restorer line, 76107, with pollen fertility restored up to 96%, was selected. This restorer line had large spikes, plant height of 90 cm, late-maturity, and susceptibility to powdery mildew. V. P. M. – Moisson 4216.1712, a line with resistance to powdery mildew, was crossed with 76107. R17 was selected from this cross and has moderate pollen fertility restoration ability and resistance to powdery mildew.

A combination of 76107//983/Kansas was made in 1982. The parental line from 983/Kansas was an early maturing line with large spike, many florets per spike and slender peduncle. A bulk selection method for the top spikelets was used in the F_2 and one plant was selected in F_3. The average fertility restoration of the F_1 hybrid in a test cross of this plant was 81.9%. Twelve F_6 breeding lines were derived from this plant. Fertility restoration of these lines varied from 81.5 to 96.1%. Seeds of two of these lines with highest restoring ability and similar plant type were composited and designated R16. R16 has the strongest and most stable fertility restoration ability of all the R-lines selected in the program. The average fertility of its hybrids test-crossed with four A-lines over 4 years was the same as the commercial common variety (Zhou et al., 1999, 2000). R16 was very susceptible to powdery mildew and scab and had small kernels. The kernel weight was only 33 mg. With these characters R16 was not suitable as a parent to produce hybrid wheat with high and stable yields (Zhou et al., 2000).

TL365 had partial restoration ability and was resistant to powdery mildew. Fertile plants from the cross of UP301A/Aining//TL365 were crossed with the fertility restorer line, Okuni (CIMMYT). Several restorer lines such as 89-3871 with resistance to powdery mildew were selected from progeny of the cross.

To improve resistance to powdery mildew, R16 was used as female parent in crosses with other fertility restorer lines, such as 89-3871, which carried the resistance genes from TL365. A series of new fertility restorer lines, including R18 were selected from these crosses. R18 maintained restoring ability similar to R16, had dominant resistance to powdery mildew, and better agronomic characters than R16. The average fertility restoration ability of R18 over 4 different B-lines was 90.2% and over four different years was 88.9% (Zhou et al., 1999).

Maiyou no. 5 is a F_1 hybrid obtained from the cross ND36/R18. ND36 is a dwarf B-line with *Rht3* height reducing gene, originating from Tom Thumb, a landrace from Tibet. In a yield test in 1996, Maiyou no. 5, yielded 8.6 t/ha which was 22.3% more than the check variety Yangmai 158. It was entered in the registration test for new wheat varieties in Jiangsu province in 1997–1998. Maiyou no. 5 ranked first in the test, and its grain yield was 13.2% greater than Yangmai 158 (Zhou et al., 2000).

Although the improved R16 restorer lines, such as R18, had strong resistance to powdery mildew, their kernel weight was similar to that of R16. To increase kernel weight, a number of crosses, including R17/PH82-2-2 were made in 1990. After continuous selection for increased kernel size and fertility restoration for 7 years, R9034, a new restoring line with large kernel size (39 mg) and strong restoring ability was obtained from the cross R17/PH82-2-2. The average fertility restoration ability of R9034 in 1996 and 1998 was 86.2% (unpublished data).

Mapping population and fertility evaluation

F_1 plants of ND36/R18 and ND36/R9034 were planted in the greenhouse, and for each cross ten heads from ten plants were bagged before flowering. Selfed kernels were mixed and 200 randomly selected F_2 kernels were planted in plastic trays, and 142 plants were used for fertility evaluation. At the two-leaf stage, a 4-cm long piece of leaf tissue was harvested separately from each seedling for DNA isolation. Plants were then transferred to a vernalization chamber for 8-weeks of vernalization at 4 °C with a 16 h photoperiod. The F_2 plants were transplanted into 15 cm pots and grown in a greenhouse at 25 °C with 16 h photoperiod. The heads from each F_2 plant were bagged before flowering, and fertility was investigated at maturity. The main head fertility was taken as the fertility of each single F_2 plant. To produce F_3 plants of ND36/R18, about 20 selfed kernels from each fertile F_2 plant were germinated. The fertility of 15 $F_{2:3}$ plants were evaluated in the greenhouse. The average fertility of these 15 $F_{2:3}$ plants in the progeny test was used to represent the fertility of the original F_2 plant. Thus, the evaluation of fertility for each F_2 plant of ND36/R18 population was based on two sources of data: the main head fertility of the single F_2 plant and the average fertility of the main heads of 15 $F_{2:3}$ plants (except for sterile F_2 plants). The fertility of a single main spike of F_2 individual plants was used for interval analysis of association between fertility and SSR markers on chromosome 1B.

DNA isolation

The harvested plant tissue was frozen in liquid nitrogen and ground with a small plastic pestle in a 2 ml

centrifuge tube. Extraction buffer (0.5 M NaCl, 0.1 M Tris–HCl, pH 8.0, 50 mM EDTA, 0.84% (w/v) sodium dodecyl sulfate, and 3.8 g/l sodium bisulfate added freshly before use, pH adjusted to 8.0 with NaOH) was heated to 65 °C, and about 1 ml of the buffer was quickly added to the frozen tissue and incubated at 65 °C for 30 min. About 0.5 ml mixture of chloroform: isoamyl alcohol (24:1) was then added, mixed vigorously, and centrifuged at 5000 rpm for 15 min. The upper phase was transferred carefully into a new tube, and DNA was precipitated with 2.5 volumes of cold 95% (v/v) ethanol. The precipitated DNA was washed in 70% (v/v) ethanol twice, dried in TE buffer, and quantified using a Beckman DU-7 Spectrophotometer.

Fertility calculation

Fertility restoration (percentage seed set) was estimated by dividing total number of viable seeds in the two lateral florets of each spikelet by total number of normally developed lateral florets in the spike and multiplying by 100.

SSR primer sources

A total of 678 pairs of SSR primers were synthesized by Integrated DNA Technologies (Coralville, IA) including 130 Xgwm SSR markers from Röder et al. (1998), 48 pairs of Xgdm SSR markers from Pestsova et al. (2000), 370 pairs of Xbarc SSR markers from Dr. P. B. Cregan in USDA-ARS, Beltsville, MD 20705, USA, 66 pairs of Xwmc SSR primers from Gupta et al. (2002), 22 pairs of EST-derived SSR markers from Eujayl et al. (2002), and 42 Xpsp SSR markers provided by Dr. M. D. Gale, John Innes Center, UK.

PCR reaction and data analysis

PCR reactions were performed as described by Röder et al. (1998) in a MJ Research PTC100 Thermal Cycler (MJ Research Inc., Waltham, MA 02451) starting with 3 min at 94 °C, then 40 cycles of 30 s at 94 °C, 30 s for annealing, and 30 s at 72 °C, with a final extension of 5 min at 72 °C. PCR products were separated on 2.5–3.0% agarose gel using 180 V for 1–2 h. Gels were stained with ethidium bromide, and visualized and photographed under UV light. Linkage of the SSR markers was analyzed by using Mapmaker/Exp, version 3.0 for the PC (Lander et al., 1987). SAS V8.0 (SAS Institute Inc., NC 27513, USA, 2000) was used for variance and regression analysis.

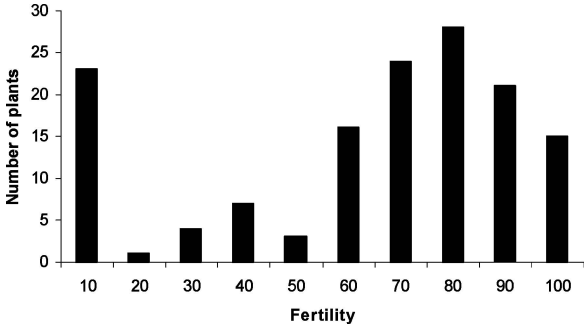

Figure 2. Distribution of fertility of 142 F_2 plants in ND36/R18 population.

Interval analysis was performed using Qgene software (Nelson, 1997).

Results

Fertility distribution in two F_2 populations

Fertility frequency distribution of F_2 plants in ND36/R18 and ND36/R9034 are shown in Figures 2 and 3, respectively. The two populations have similar distribution patterns with a continuous distribution of fertility from 0 to 100%. A portion of plants are completely sterile in both populations.

SSR markers associated with fertility restoration in two populations

Among 678 analyzed SSR markers, 112 and 98 were polymorphic between ND36 and R18, and between ND36 and R9034, respectively. These markers were further analyzed in the two F_2 populations. All SSR markers significantly associated with fertility

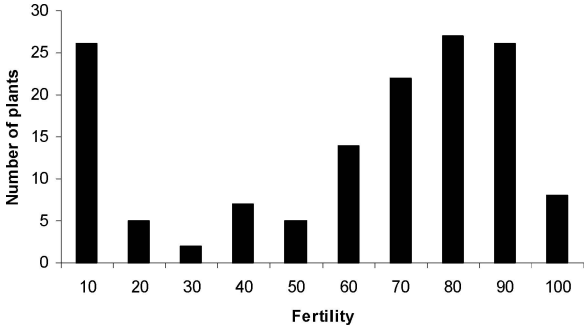

Figure 3. Distribution of fertility of 142 F_2 plants in ND36/R9034 population.

Table 1. SSR markers associated with fertility restoring QTL in R18 and R9034

Population and markers	Chromosome	*p* value	R^2 (%)	Fertility of F_2 plants		
				R (%)	*H* (%)	*A* (%)
ND36/R18						
Xbarc207	1B	<0.0001	38.2	74.0	66.4	27.5
Xgwm131	1B	<0.0001	35.0	71.2	66.9	30.1
Xbarc61	1B	<0.0001	29.5	71.2	64.2	26.5
Xgwm582	1B	<0.001	11.1	70.3	55.7	42.4
Xbarc268	1B	0.0016	10.4	68.1	54.7	44.5
Xbarc330	5A	0.014	4.7	64.5	–	51.6
ND36/R9034						
Xbarc207	1B	<0.0001	34.0	69.9	57.3	26.3
Xgwm131	1B	<0.0001	32.3	69.2	57.3	28.8
Xbarc61	1B	<0.0001	30.0	68.5	56.9	33.5
Xgwm582	1B	0.0004	12.4	70.3	51.4	39.1
Xgdm130	7D	0.0238	4.2	61.5	–	48.7

R: homozygotes for maker allele from restorer line, R18 or R9034; H: heterozygotes for marker allele; A: homozygotes for marker allele from sterile line, ND36.

restoration in one or both of the populations are listed in Table 1. A group of SSR markers on chromosome 1B were significantly associated with fertility restoration in both F_2 populations. The best marker, Xbarc207, explained 38.2 and 34.0% of total phenotypic variation in the two populations, respectively. Three genotypic groups based on marker Xbarc207 had significantly different fertility percentages (Figure 4). Most homozygotes for the allele from the restorer lines (based on the marker) have fertility over 70%, heterozygotes have fertility over 60%, and most plants homozygous for the allele from ND36 have fertility less than 20% (Figure 4). The average fertility of the three genotypic groups of F_2 plants separated by all listed 1B markers in Table 1 showed that the 1B fertility restoration gene is partially dominant. There is significant difference between fertility values of homozygotes for the allele from R18 or R9034 and heterozygotes in the two populations.

Both R18 and R9034 were derived from Primepi, which carries a major fertility restoration gene, *Rf3*, on chromosome 1BS (Ma & Sorrels, 1995; Kojima et al., 1997; Ahmed et al., 2001). Based on the fact that the same SSR markers were significantly associated with fertility restoration in both populations, it is probable that both R18 and R9034 carry *Rf3*. Based on interval analysis (Figure 5) the three SSR markers most closely linked to *Rf3* are Xbarc207, Xgwm131, and Xbarc61. These markers may be useful for marker-assisted selection of new fertility restorer lines.

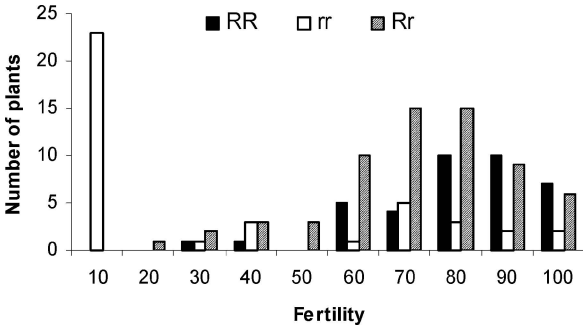

Figure 4. Frequency distribution of fertility of 142 F_2 plants separated into three genotypic groups based on marker Xbarc207. RR: R18 allele homozygotes, Rr: heterogotes, rr: ND36 allele homozygotes.

In addition to the *Rf3* gene there may be genes with minor effects on fertility restoration in the two restorer lines, R18 and R9034. In population ND36/R18, Xbarc330, a SSR marker on chromosome 5A, was significantly associated with fertility restoration. In ND36/R9034, Xgdm130, a marker on 7D, was significantly associated with fertility restoration. Xbarc330 and Xgdm130 are dominant markers. They only amplify one band from R18 or R9034.

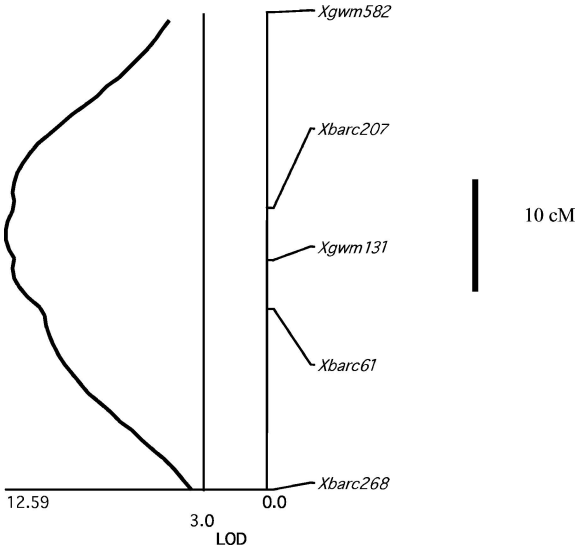

Figure 5. Interval analysis of fertility restoration associated with SSR markers on chromosome 1B in F_2 population of ND36/R18. Curve indicates LOD value distribution over the chromosome region.

Correlation between fertility of F_2 plants scored from single plants and average values from the progeny test of 15 $F_{2:3}$ plants

There was a significant correlation ($r = 0.8149$, $p = 0.038$) between fertility of single F_2 plants and average fertility of 15 $F_{2:3}$ plants in population ND36/R18; however, all 1B SSR markers had a higher coefficient of determination using $F_{2:3}$ progeny fertility data than with single plant fertility data (Table 2). Compared to using single plant fertility data, fertility values of plants homozygous for the allele from R18 based on 1B SSR markers were all increased about 10% when using progeny fertility data, but there were not similar changes in the other two genotypic groups. This indicates that the fertility of plants homozygous for the *Rf3* gene was underestimated when using single plant fertility data.

Discussion

PCR-based molecular markers for Rf3 and marker-assisted selection

Selection of fertility restorer lines is much more complicated than that of common wheat varieties because of the necessity for additional selection of high and stable fertility restoration in breeding restorer lines (Zhou et al., 1999). To maintain fertility restoration genes, beginning with the F_3 generation, plants selected from a segregating population based on agronomic traits must be test-crossed with different sterile lines. Selection in later generation has to be done after fertility data are available. This increases the work load during the short period from flowering to maturity. The whole process for breeding a fertility restorer line is 3–5 years longer than for breeding a common variety.

The *Rf3* gene is one of the few Rf genes that has been associated with molecular markers. Kojima et al. (1997) mapped the *Rf3* gene with RFLP markers Xcdo388 and Xabc156 on chromosome 1BS using a BC_3F_2 population of 125 plants. Ahmed et al. (2001) identified an Rf gene linked to the RFLP marker XksuG9c on 1BS. This gene most likely corresponds to the *Rf3* gene identified by Kojima et al. (1997) since the same parental lines were used to produce the mapping populations. Ma & Sorrels (1995) located *Rf3* in

Table 2. Comparisons of coefficient of determination and marker allele effect of 1B SSR markers linked to fertility restoration in ND36/R18 based on fertility values scored on single F_2 plants and 15 $F_{2:3}$ plants

				Marker allele					
		R^2		*R* (%)		*H* (%)		*A* (%)	
Marker	Chromosome	F_2 (%)	$F_{2:3}$ (%)	F_2	$F_{2:3}$	F_2	$F_{2:3}$	F_2	$F_{2:3}$
Xbarc207	1B	38.2	40.7	74.0	84.5	66.4	67.5	27.5	29.5
Xgwm131	1B	35.0	35.6	71.2	81.3	66.9	66.9	30.1	31.1
Xbarc61	1B	29.5	37.7	71.2	80.9	64.2	65.7	26.5	23.5
Xgwm582	1B	11.1	20.2	70.3	81.5	55.7	57.2	42.4	41.5
Xbarc268	1B	10.4	17.8	68.1	78.7	54.7	56.3	44.5	42.8

R: homozygotes for marker allele from restorer line, R18 or R9034; H: heterozygotes for marker allele; A: homozygotes for marker allele from sterile line, ND36.

Primepi to 1BS, 23 cM from Nor and associated with RFLP markers Xcdo442-1B and Xbcd249-1B.

For the SSR makers linked to major fertility QTL in our mapping population, Xgwm582 is located in chromosome 1BS (Röder et al., 1998); Xgwm131 and Xbarc61 are located in chromosome 1BL in ITMI mapping population (Röder et al., 1998; J. Shi and R. Ward, personal communication). However, Xbarc61 was physically mapped on 1BS with Chinese Spring deletion lines and ditelosomic lines (J. Shi and R. Ward, personal communication). The location of Xbarc207 in ITMI mapping population was not determined due to a lack of polymorphism between two parents (J. Shi and R. Ward, personal communication). It is most likely that Xbarc207 is located on chromosome 1BS and *Rf3* gene is located either between Xgwm582 and Xbarc207 or between Xbarc207 and Xgwm131 but very close to Xbarc207 in our mapping population. This can be clarified if there are molecular markers mapped between Xbarc207 and Xgwm582 and they can explain more variation of fertility restoration. Since we did not map previous identified RFLP markers linked to *Rf3* in our mapping population, we could not construct a linkage map including these RFLP markers for a better estimation of distance between *Rf3* and SSR markers in our mapping population.

RFLP markers are not user-friendly, time-consuming, and more costly compared to PCR-based markers. SSR markers associated with the *Rf3* gene on chromosome 1B were identified in current study. A significant allele effect detected by the markers showed that *Rf3* is a major gene conferring fertility restoration in two elite R-lines. These SSR markers should be useful in marker-assisted selection of new R-lines and reduce the time required for test-crossing and thus shorten the breeding process.

Two minor fertility restoration genes

One minor fertility gene was identified in R18 on chromosome 5A by marker Xbarc330. In a previous study, Ma and Sorrels (1995) identified an *Rf* gene on chromosome 5AL in Primepi, which was linked to RFLP markers Xbcd183, Xbcd876, and Xcdo786. Xbarc330 was not polymorphic between ND36 and R9034 so we could not confirm if R9034 carried this gene.

Marker Xgdm130 on 7D was associated with fertility restoration in R9034. According to the "Catalogue of Gene Symbols for Wheat" (McIntosh et al., 1998), an *Rf* gene on 7D has been designated *Rf2*, based on a study by Livers (Livers, 1964). Panayotov et al. (1988) also showed that an *Rf* gene is present 7D. No molecular markers linked to fertility restoration genes on chromosome 7D have been reported to date.

Due to a lack of polymorphic SSR markers in the two regions containing Xbarc330 on 5A and Xgdm130 on 7D, we could not conduct an interval analysis of these two minor fertility restoration genes to obtain an accurate estimation of their restoring ability. We believe they are important for the stable and strong restoring ability of R18 and R9034. In the future additional PCR-based molecular markers in these regions of chromosomes 5A and 7D would aid in determining the effects of these genes.

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